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The barley scald pathogen *Rhynchosporium secalis* is closely related to the discomycetes *Tapesia* and *Pyrenopeziza*

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Rhynchosporium secalis causes an economically important foliar disease of barley, rye, and other grasses known as leaf blotch or scald. This species has been difficult to classify due to a paucity of morphological features; the genus *Rhynchosporium* produces conidia from vegetative hyphae directly, without conidiophores or other structures. Furthermore, no teleomorph has been associated with *R. secalis*, so essentially nothing is known about its phylogenetic relationships. To identify other fungi that might be related to *R. secalis*, the 18S ribosomal RNA gene and the internal transcribed spacer (ITS) region (ITS1, 5.8S rRNA gene, and ITS2) were sequenced and compared to those in databases. Among 31 18S sequences downloaded from GenBank, the closest relatives to *R. secalis* were two species of *Graphium* (hyphomycetes) and two other accessions that were not identified to genus or species. Therefore, 18S sequences were not useful for elucidating the phylogenetic relationships of *R. secalis*. However, analyses of 76 ITS sequences revealed very close relationships among *R. secalis* and species of the discomycete genera *Tapesia* and *Pyrenopeziza*, as well as several anamorphic fungi including soybean and Adzuki-bean isolates of *Phialophora gregata*. These species all clustered together with 100% bootstrap support. On the basis of these results, the teleomorph of *R. secalis*, if it exists, most likely will be a small apothecium produced directly on dead, infected host tissue. The ITS analysis also indicated that higher-level classifications within the discomycetes need to be revised, and that *Tapesia* and *Pyrenopeziza* probably do not belong in the *Dermateaceae*.

INTRODUCTION

Rhynchosporium secalis causes an economically important disease of barley known as leaf blotch or, in the USA and Canada, as scald. Leaf blotch occurs throughout the world wherever barley is grown and can cause yield losses of 35% or more (Shipton, Boyd & Ali 1974). In addition to cultivated barley (*Hordeum vulgare*), *R. secalis* infects rye and several wild grass species (Caldwell 1937).

No teleomorph has been described for *R. secalis* and essentially nothing is known about its phylogenetic relationships. Conidia of *R. secalis* typically are two celled and hyaline with a characteristic beak at one end, and are produced on mycelia directly with no true conidiophores (Caldwell 1937). Small, single-celled microconidia of unknown function sometimes are produced from flask-like branches of older mycelia (Skoropad & Grinchenko 1957). Lesions are oval or lens shaped, light grey in the centre with a characteristic dark margin, and are produced from a subcuticular stroma on leaf or stem tissue with no other morphological features (Caldwell 1937). This relative paucity of morphological characters makes an accurate classification of *R. secalis* difficult.

The genus *Rhynchosporium* contains only two accepted species. In addition to *R. secalis*, a second species, *R. orthosporum*, was described by Caldwell (1937). This species is very similar to *R. secalis* morphologically, but it has conidia that are uniformly cylindrical rather than beaked. Instead of barley, the host of *R. orthosporum* is orchardgrass (*Dactylis glomerata*). A third species sometimes placed in the genus *Rhynchosporium*, *R. alismatis*, infects *Alisma*, *Sagittaria*, and other hosts in the *Alismataceae* and is being developed as a potential biocontrol agent for weedy members of that family (Cother & Gilbert 1994, Cother 1999). However, because this species produces conidia from conidiophores and lacks a superficial stroma, Caldwell (1937) excluded it from *Rhynchosporium*. So far, none of these species has been studied phylogenetically and it is not known if the genus *Rhynchosporium* is monophyletic.

The purpose of this study was to identify fungi related to *R. secalis* through phylogenetic analysis of the 18S ribosomal RNA gene and internal transcribed

spacer (ITS) region of the ribosomal DNA (ITS1, 5.8S rRNA gene, ITS2). These regions were chosen to include both slowly and rapidly evolving sequences, and because they are the largest components of existing databases which should increase the probability of identifying related species. Specific objectives were to identify which teleomorph and anamorph genera are related to *R. secalis*, to predict the probable teleomorph association of the genus *Rhynchosporium*, and to test whether *R. secalis* and *R. orthosporum* are part of a monophyletic group.

MATERIALS AND METHODS

Sources and culturing of isolates

Six isolates of *Rhynchosporium secalis* were obtained from five countries in Europe and North America (Table 1). The isolates from Finland, Norway, and the USA were studied by Salamati *et al*. (2000); those from France and the UK have not been analysed previously. Isolates were purified from infected barley leaves and cultured on potato-dextrose or lima-bean agar plates (McDonald, Zhan & Burdon 1999, Salamati *et al*. 2000). Following 10–14 d growth, 2 ml of sterile water was added to each Petri dish and *R. secalis* spores were scraped from the surface of the agar using a sterilised glass microscope slide. Then 80 μl of the spore solution was added to 2 ml screw-top Nunc cryovials filled with a 9:1 mixture of dried silica gel (average particle size 0.13 mm diam) and indicator silica gel (2 mm diam). Cryovials were placed at −80 °C for long-term storage at ETH, Zurich, with duplicates deposited at CBS. One ml of the spore solution was added to 40-ml flasks containing potato-dextrose broth to obtain tissue for DNA extraction. Flasks contained 50 μg ml^{-1} of kanamycin and were shaken at 100 rev min^{-1} at 15 ° for 3–4 weeks. Mycelia were harvested by centrifugation for 10 min at 10000 rev min^{-1} in 15-ml polypropylene tubes, lyophilised for 48 h and stored at −80 °.

DNA extraction, cloning, and sequencing

DNA was extracted from lyophilised tissue as described by McDonald *et al*. (1999) and was quantified with a Hoefer DyNAQuant 2000 fluorometer (Hoefer Scientific Instruments, San Francisco, CA). The complete ITS region (ITS1, 5.8S rRNA gene, ITS2) of each isolate was amplified with primers ITS4 and ITS5 of White *et al*. (1990). Amplification in a Perkin–Elmer 9600 thermal cycler (Perkin–Elmer, Foster City, CA), cloning of PCR products with the TA cloning kit (Invitrogen Corp., Carlsbad, CA), and sequencing the inserts with the ThermoSequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ) on an ALFexpress automated DNA sequencer (Amersham Pharmacia Biotech, Piscataway, NJ) were as described previously (Goodwin, Dunkle & Zismann 2001, Goodwin & Zismann 2001). Each clone was sequenced in both directions with the M13 reverse and M13-40 primers. From three to six clones per isolate were sequenced to detect and remove possible errors caused by PCR amplification.

The 18S rRNA gene was sequenced as described above except it was cloned in overlapping segments with primer pairs NS1/NS4, NS3/NS6, and NS5/NS8 (White *et al*. 1990). These segments were then assembled into the complete 18S rRNA gene sequence with the Contig Manager of MacDNASIS (Hitachi Software, San Francisco, CA).

Assembling the 18S and ITS sequence databases

To identify species that might be closely related, a BLAST (Altschul *et al*. 1997) search was performed on the 18S rRNA gene sequence from isolate 788 of *R. secalis*. Sequences of 31 accessions with high similarity to the *R. secalis* sequence were downloaded from GenBank and added to the 18S database (Table 2).

For the ITS database, sequences were obtained from four sources (Table 3) in addition to those generated *de novo*. Most of the sequences were downloaded from GenBank following a BLAST search as described above. Sequences for two species of *Tapesia* (anamorph *Ramulispora*) and two species of *Ramulispora* (syn. *Pseudocercosporella*) with no known teleomorph (Stewart *et al*. 1999) were downloaded from TreeBASE (available on line from the Harvard University Herbaria), converted into FASTA format, and added to the database. The ITS sequences for two isolates of *Pyrenopeziza brassicae* were kindly provided by Simon Foster (IACR-Rothamsted, Harpenden). Finally, sequences for one isolate of *R. secalis* and the related species *R. orthosporum* were taken from a recent

Table 1. Collection information for the six barley isolates of *Rhynchosporium secalis* used for phylogenetic analysis.

Isolate	Location	Country	Cultivar	Year	Collector
763	Suffolk	UK	Pipkin	1997	John-Bryan Speakman
788	Cane Hemont	France	Maeva	1997	John-Bryan Speakman
FI12-63	Jokoinen	Finland	Kymppi	1996	Marja Jalli
NKT 12	Stjordal	Norway	Tyra	1996	Saideh Salamati
R157	Davis, CA	USA	CC II[a]	1986	Bruce McDonald
R164	Davis, CA	USA	CC II[a]	1986	Bruce McDonald

[a] Composite Cross II.

Table 2. Summary information for 32 isolates included in the 18S ribosomal RNA gene sequence database[a].

Species or accession name	Family	Order	GenBank accession no.
Arthrobotrys conoides	–	–	AJ001983
A. dactyloides	–	–	AJ001997
A. musiformis	–	–	AJ001985
A. oligospora	–	–	AJ001986
A. robusta	–	–	AJ001988
Arthrocladiella mougeotii	*Erysiphaceae*	*Erysiphales*	AB033477
Aureobasidium pullulans	–	–	M55639
Blumeria graminis f. sp. *bromi*	*Erysiphaceae*	*Erysiphales*	AB033476
Botryosphaeria ribis	*Botryosphaeriaceae*	*Dothideales*	U42477
Bulgaria inquinans	*Leotiaceae*	*Leotiales*	AJ224362
Byssoascus striatosporus	*Myxotrichaceae*	*Onygenales*	AB015776
Dark septate endophyte[b]	–	–	AF168167
Dothidea insculpta	*Dothideaceae*	*Dothideales*	U42474
Erysiphe orontii	*Erysiphaceae*	*Erysiphales*	AB033483
Euascomycetes sp.[b]	–	–	AB016175
Geoglossum nigritum	*Geoglossaceae*	*Leotiales*	AF113716
Graphium rubrum	–	–	AB007660
G. silanum	–	–	AB007661
Hortaea werneckii	–	–	Y18700
Lecidea fuscoatra	*Lecideaceae*	*Lecanorales*	AF088239
Leotia lubrica	*Leotiaceae*	*Leotiales*	L37536
Leptosphaeria maculans	*Leptosphaeriaceae*	*Pleosporales*[c]	U04233
Leveillula taurica	*Erysiphaceae*	*Erysiphales*	AB033479
Monilinia laxa	*Sclerotiniaceae*	*Leotiales*	Y14210
Mycosphaerella mycopappi	*Mycosphaerellaceae*	*Dothideales*	U43449
Oidiodendron tenuissium	Anamorphic *Myxotrichaceae*	*Onygenales*	AB015787
Pleospora rudis	*Pleosporaceae*	*Pleosporales*[c]	U00975
Pseudogymnoascus roseus	*Myxotrichaceae*	*Onygenales*	AB015778
Rhynchosporium secalis	–	–	AY038583
Sclerotinia sclerotiorum	*Sclerotiniaceae*	*Leotiales*	L37541
Spathularia flavida	*Geoglossaceae*	*Leotiales*	Z30239
Sphaerotheca cucurbitae	*Erysiphaceae*	*Erysiphales*	AB033482

[a] Taxonomy mostly according to Hawksworth *et al.* (1995).
[b] These accessions were not identified to species in GenBank.
[c] Listed as *Dothideales* by Hawksworth *et al.* (1995) but as *Pleosporales* by Eriksson & Winka (1998).

publication (Lee, Tewari & Turkington 2001). Because these sequences were not available in public databases, they were entered manually based on the published alignment.

Sequence alignment and phylogenetic analyses

Sequences obtained from different sources did not always begin and end at the same nucleotide positions. To facilitate comparisons, all 18S sequences were trimmed to the region corresponding to bases 92–1704 of the *R. secalis* isolate 788 18S sequence (GenBank accession no. AY038583). Alignment of 18S sequences was accomplished with the Do Complete Alignment command of ClustalX (Thompson *et al.* 1997) and the default settings for gap opening and extension penalties.

All ITS sequences were trimmed to include the complete ITS1, 5.8S rRNA gene, and ITS2 sequences. Seven bases each of the 18S and 26S gene sequences were included at the beginning and end of most sequences, respectively, to aid alignment. The aligned region corresponds to bases 348–904 of the ITS sequence of *R. secalis* isolate 763 (GenBank accession no. AF384677).

The ITS sequences were aligned through a three-step process with the profile mode of ClustalX (Thompson *et al.* 1997) as described by Goodwin *et al.* (2001). A preliminary simultaneous multiple alignment of all sequences was performed to identify groups of closely related taxa. Then a separate alignment was performed for each group and saved as a different profile. Finally, the profiles were aligned to each other using the preliminary dendrogram as a guide. Sequences that did not cluster with any of the others in the initial step were aligned as separate profiles. Gap opening and extension penalties were reduced when necessary to aid alignment of shorter sequences with those that had large insertions near the 5′ end of ITS1. Each profile was checked by eye and edited manually if necessary before proceeding to the next step. Following alignment, genetic distances among all isolates were calculated, and a neighbour-joining tree was prepared with the Draw N-J Tree option of ClustalX. This option uses Kimura's two-parameter method for estimating evolutionary distances (Kimura 1980) and implements the neighbour-joining algorithm of Saitou & Nei (1987). Bootstrap analysis (1000 replications) was performed with the Bootstrap N-J Tree option of ClustalX, and the final trees were

Table 3. Summary information for 76 isolates included in the internal transcribed spacer sequence database[a].

Species	Isolate	Family	Order	Host genus or substrate	GenBank accession no.
Arachnopeziza aurata	JHH2210 NYS	*Hyaloscyphaceae*	*Leotiales*	–	U57496
Arthroderma benhamiae	UAMH 7339	*Arthrodermataceae*	*Onygenales*	*Homo*	AF170467
A. gypseum	CBS 161.69	*Arthrodermataceae*	*Onygenales*	*Homo*	AF168129
Ascocalyx abietina	ATCC 28379	*Leotiaceae*	*Leotiales*	*Pinus*	AF260815
Chrysosporium georgiae	CBS 272.66	Anamorphic *Arthrodermataceae*	*Onygenales*	*Soil*	AJ007844
Cistella grevillei	JHH1602 NYS	*Hyaloscyphaceae*	*Leotiales*	–	U57089
Cyclaneusma minus	–	*Rhytismataceae*	*Rhytismatales*	*Pinus*	AF013222
C. niveum	CBS 495.73	*Rhytismataceae*	*Rhytismatales*	*Pinus*	AF013223
Dactylella lobata	CBS 228.54	–	–	*Nematodes*	U51958
Dark septate endophyte[b]	DS16b	–	–	*Ranunculus*	AF168783
Dermea cerasi	CBS 136.46	*Dermateaceae*	*Leotiales*	–	AF141159
Ericoid mycorrhizal sp.[b]	Sd9	–	–	*Quercus*	AF269067
Gremmeniella abietina	CF-87-0036	*Leotiaceae*	*Leotiales*	*Pinus*	U72257
G. abietina	CF-87-0061	*Leotiaceae*	*Leotiales*	*Abies*	U72259
G. laricina	M1041	*Leotiaceae*	*Leotiales*	*Larix*	U72262
Hymenoscyphus ericae	21	*Leotiaceae*	*Leotiales*	*Cephaloziella*	AF069439
H. ericae	BH	*Leotiaceae*	*Leotiales*	*Cephaloziella*	AF069440
H. ericae	UBCtra241	*Leotiaceae*	*Leotiales*	*Gaultheria*	AF149068
H. ericae	UBCtra274	*Leotiaceae*	*Leotiales*	*Gaultheria*	AF149069
Kabatina thujae	CBS 238.66	–	–	*Thuja*	AF013226
Lachnellula calyciformis	JHH 4622 NYS	*Hyaloscyphaceae*	*Leotiales*	–	U59145
Lachnum clandestinum	JHH 4676 NYS	*Hyaloscyphaceae*	*Leotiales*	–	U58636
L. euterpes	Cantrell PR147	*Hyaloscyphaceae*	*Leotiales*	*Euterpe*	U58640
L. spartinae	RTH 1078 GAM	*Hyaloscyphaceae*	*Leotiales*	–	U58639
Lophodermium pinastri	ATCC 28347	*Rhytismataceae*	*Rhytismatales*	*Pinus*	AF013224
Neofabraea[c] *alba*	CBS 452.64	*Dermateaceae*	*Leotiales*	–	AF141190
N.[c] *malicorticis*	–	*Dermateaceae*	*Leotiales*	–	AF141189
N.[c] *malicorticis*	CBS 141.22	*Dermateaceae*	*Leotiales*	*Malus*	AF141161
Oat root associated[b]	00015	–	–	*Avena*	AJ246143
Oat root associated[b]	00036	–	–	*Avena*	AJ246144
Oat root associated[b]	0004	–	–	*Avena*	AJ246141
Oat root associated[b]	0006	–	–	*Avena*	AJ246142
Pezicula carpinea	CBS 921.96	*Dermateaceae*	*Leotiales*	*Carpinus*	AF141197
P. cinnamomea	CBS 236.97	*Dermateaceae*	*Leotiales*	*Acer*	AF141185
P. cinnamomea	CBS 290.39	*Dermateaceae*	*Leotiales*	–	AF141184
P. cinnamomea	CBS 625.96	*Dermateaceae*	*Leotiales*	–	AF141186
P. cinnamomea	CBS 778.95	*Dermateaceae*	*Leotiales*	*Larix*	AF141187
P. heterochroma	CBS 199.46	*Dermateaceae*	*Leotiales*	*Alnus*	AF141167
P. ocellata	CBS 267.39	*Dermateaceae*	*Leotiales*	*Salix*	AF141181
P. sp.	CBS 101.96	*Dermateaceae*	*Leotiales*	–	AF141173
P. sporulosa	CBS 224.96	*Dermateaceae*	*Leotiales*	*Larix*	AF141172
Phialophora gregata	46906	–[d]	–	*Phaseolus*	U66731
P. gregata	G3	–[d]	–	*Glycine*	U66730
Pseudogymnoascus roseus	UAMH 9163	*Myxotrichaceae*	*Onygenales*	*Abies*	AF062819
Pyrenopeziza brassicae	CRB	*Dermateaceae*[e]	*Leotiales*	*Brassica*	Simon Foster
P. brassicae	jh26	*Dermateaceae*[e]	*Leotiales*	*Brassica*	Simon Foster
Ramulispora aestiva	RAE22	–	–	*Triticum*	S474[f]
R. anguioides	RAN45	–	–	*Triticum*	S474[f]
Rhynchosporium orthosporum	CBS 698.79	–	–	*Dactylis*	Lee *et al.* (2001)
R. secalis	763	–	–	*Hordeum*	AF384677
R. secalis	788	–	–	*Hordeum*	AF384678
R. secalis	Fl12-63	–	–	*Hordeum*	AF384681
R. secalis	NKT 12	–	–	*Hordeum*	AF384682
R. secalis	R157	–	–	*Hordeum*	AF384679
R. secalis	R164	–	–	*Hordeum*	AF384680
R. secalis	RS 20	–	–	*Hordeum*	Lee *et al.* (2001)
Salal mycorrhizal[b]	UBCtra323	–	–	*Gaultheria*	AF149083
Salal mycorrhizal[b]	UBCtra43	–	–	*Gaultheria*	AF149082
Salal mycorrhizal[b]	UBCtra51	–	–	*Gaultheria*	AF149086
Salal mycorrhizal[b]	UBCtra69	–	–	*Gaultheria*	AF149084
Salal root associated[b]	UBCtra180	–	–	*Gaultheria*	AF149071
Salal root associated[b]	UBCtra264	–	–	*Gaultheria*	AF149070
Scleropezicula[g] *alnicola*	CBS 200.46	*Dermateaceae*	*Leotiales*	–	AF141168
S. sclerotiorum	1	*Sclerotiniaceae*	*Leotiales*	*Brassica*	M96382
Sclerotinia sp.	5/97-18	*Sclerotiniaceae*	*Leotiales*	*Phragmites*	AJ279480

Table 3. (*cont.*)

Species	Isolate	Family	Order	Host genus or substrate	GenBank accession no.
Sclerotinia borealis	SB	*Sclerotiniaceae*	*Leotiales*	–	AF067644
Solenopezia solenia	JHH4169 NYS	*Hyaloscyphaceae*	*Leotiales*	–	U57991
Tapesia[h] *acuformis*	RAC44	*Dermateaceae*[e]	*Leotiales*	*Secale*	S474[f]
T.[h] *yallundae*	RH26	*Dermateaceae*[e]	*Leotiales*	*Triticum*	S474[f]
Trichophyton rubrum	ATCC 28188	Anamorphic *Arthrodermataceae*	*Onygenales*	*Homo*	AF170472
T. soudanense	UAMH 8548	Anamorphic *Arthrodermataceae*	*Onygenales*	*Homo*	AF170474
T. verrucosum	–	Anamorphic *Arthrodermataceae*	*Onygenales*	*Homo*	AF168126
Umbilicaria crustulosa	–	*Umbilicariaceae*	*Lecanorales s. lat.*	–	AF096215
U. decussata	–	*Umbilicariaceae*	*Lecanorales s. lat.*	–	AF096214
U. hyperborea	–	*Umbilicariaceae*	*Lecanorales s. lat.*	–	AF096216
U. rigida	–	*Umbilicariaceae*	*Lecanorales s. lat.*	–	AF096212

[a] Taxonomy mostly according to Hawksworth *et al.* (1995).
[b] These accessions were not identified to species in GenBank.
[c] *Neofabraea* is considered a synonym for *Pezicula* by Hawksworth *et al.* (1995).
[d] Listed as anamorphic *Magnaporthaceae* by Hawksworth *et al.* (1995).
[e] Listed as *Dermateaceae* by Hawksworth *et al.* (1995) but as *Mollisiaceae* in the GenBank taxonomy database.
[f] TreeBASE accession number.
[g] Not listed in Hawksworth *et al.* (1995) but was in the GenBank taxonomy database.
[h] Sequences are listed in TreeBASE under *Ramulispora*.

visualised and printed with Njplot (Perrière & Gouy 1996).

RESULTS

Analysis of 18S sequences

The 18S rRNA gene sequence of *Rhynchosporium secalis* isolate 788 was 2074 bases long, including the primer regions. This is longer than in most other species, due to an insertion of 298 base pairs near the 3′ end of the 18S gene sequence (bases 1757–2054). The insertion region was trimmed before alignment and was not included in the phylogenetic analysis. When the *R. secalis* 18S sequence was compared to others in GenBank with the BLAST algorithm, no extremely close matches were identified. However, sequences of 31 fungi with the highest similarity to the *R. secalis* 18S sequence were downloaded and added to the database. The final 18S database contained sequences from species in 19 teleomorph genera, representing 11 families and six orders, plus 11 species from six anamorph genera with no known teleomorph (Table 2). Two accessions were not identified to genus or species and were listed as 'dark septate endophyte' and '*Euascomycetes* sp.'.

Due to the high conservation of 18S rRNA gene sequences, alignment was accomplished in a single step without the need for manual correction. The final data set contained 1629 bases of aligned sequence.

In the neighbour-joining tree derived from the 18S data, *R. secalis*, the *Euascomycetes* sp., dark septate endophyte, and two species of *Graphium* clustered together with 100% bootstrap support (Fig. 1). The next closest cluster was a group of species with teleomorphs in the *Erysiphales*.

Three orders appeared to be polyphyletic. The *Dothideales*, *Leotiales*, and *Onygenales* each had members in two very different clusters (Fig. 1).

Analysis of ITS sequences

The sequence of the ITS region was obtained for six isolates of *Rhynchosporium secalis* from Europe and the USA (Table 1). The length of the ITS region amplified from *R. secalis* was 952 bases, including the primer regions. This is much longer than most ITS sequences, mainly due to an insertion of 298 base pairs near the 3′ end of the 18S rRNA gene (see the section on 18S sequences above). The region used for phylogenetic analysis was 557 bases long, including seven bases each of the 18S and 26S gene sequences that were retained at the beginning and end of the sequence, respectively, to aid alignment. The *R. secalis* ITS region alone was 539 bases: 233 bases for ITS1, 158 for the 5.8S gene sequence, and 148 bases for ITS2. Five sequences were identical, but the sixth (from isolate NKT 12) had an extra G in ITS1 at base 411 of the *R. secalis* isolate 763 ITS sequence (GenBank accession no. AF384677). These five sequences were identical to that published recently by Lee *et al.* (2001) except the previously published sequence was missing a G near the beginning of ITS1 (base 386 of the *R. secalis* isolate 763 ITS sequence).

Based on the results of the BLAST search, 62 sequences representing 42 species, eight families, and four orders were downloaded from GenBank and

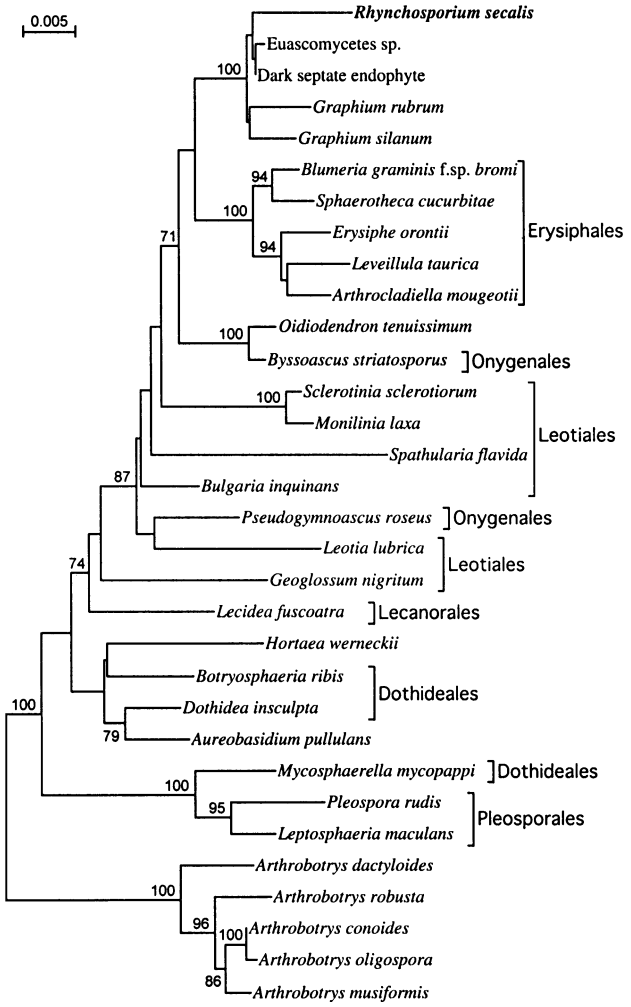

Fig. 1. Unrooted neighbour-joining tree of 32 sequences of the 18S ribosomal RNA gene from *Rhynchosporium secalis* (indicated in bold) and other fungi identified through a BLAST (Altschul *et al.* 1997) search. All bootstrap values of 70 or greater (percent of 1000 replications) are indicated, rounded to the nearest integer. The order for teleomorph genera is indicated by brackets to the right. Branch lengths are proportional to genetic distance, which is indicated by a bar at the upper left.

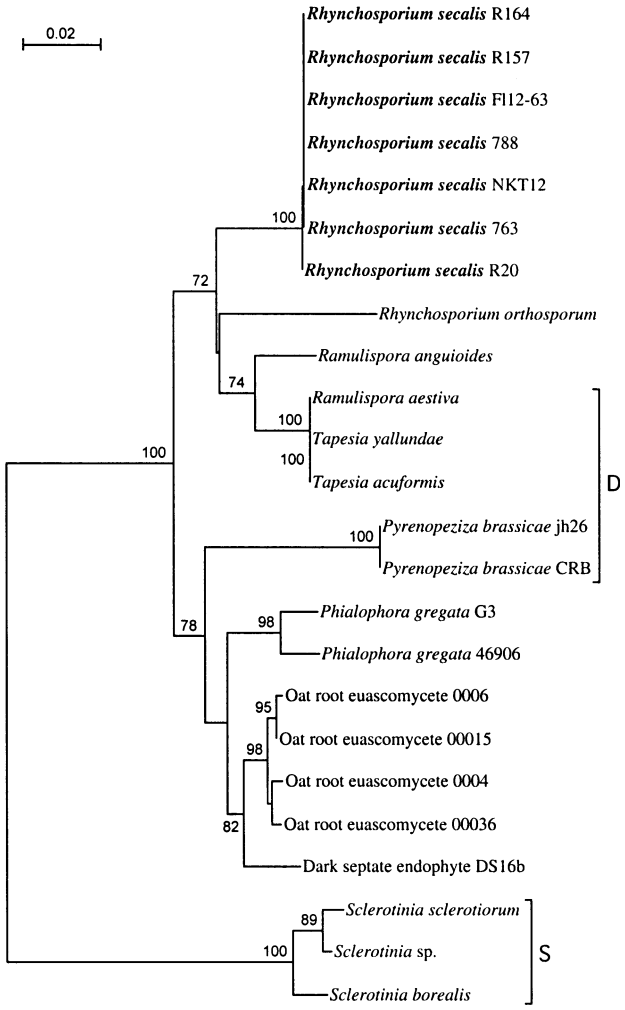

Fig. 2. Unrooted neighbour-joining tree of 21 sequences of the internal transcribed spacer region of the ribosomal DNA from *Rhynchosporium secalis* (indicated in bold) and closely related fungi identified in preliminary analyses. Sequences from three species of *Sclerotinia* were included as an outgroup. If more than one isolate of a species was analysed, isolate designations were provided after the species name. All bootstrap values of 70 or greater (percent of 1000 replications) are indicated, rounded to the nearest integer. Families of teleomorph genera according to Hawksworth *et al.* (1995) are indicated by brackets to the right: D, *Dermateaceae*; S, *Sclerotiniaceae*. Branch lengths are proportional to genetic distance, which is indicated by a bar at the upper left.

added to the database (Table 3). These included six species from the *Arthrodermataceae* (order *Onygenales*) included as an outgroup, and 12 accessions that were not identified to genus or species. With the addition of the ITS sequences of *R. secalis* and *R. orthosporum* from Lee *et al.* (2001), the four *Ramulispora* sequences from Stewart *et al.* (1999) (two listed in Table 3 as *Tapesia* species), two *Pyrenopeziza brassicae* sequences provided by Simon Foster, and the six new *R. secalis* sequences, the final database contained 76 sequences representing 49 species in 27 genera (both meio- and mitosporic), nine families, and four teleomorph orders (Table 3). Taxonomy of most species was listed as indicated by Hawksworth *et al.* (1995). However, for a few species not mentioned in Hawksworth *et al.* (1995), the taxonomy of Eriksson & Winka (1998) was followed.

Two analyses were performed. The first included all of the *R. secalis* sequences and those of the 14 other accessions that appeared most closely related to *R. secalis* in preliminary analyses. Three sequences from *Sclerotinia* species (*Sclerotiniaceae*, order *Leotiales*) were included as an outgroup. Alignment of the sequences required 17 profile steps, only three of which needed minor manual editing.

This analysis clearly indicated that *R. secalis* is very closely related to species of *Tapesia* and its anamorph *Ramulispora* (Fig. 2). The ITS sequence of *R. orthosporum* differed from that of *R. secalis* isolate 763 by 38 nucleotides and clustered between the *R. secalis* and *Tapesia*/*Ramulispora* clusters. The sequence of *T.*

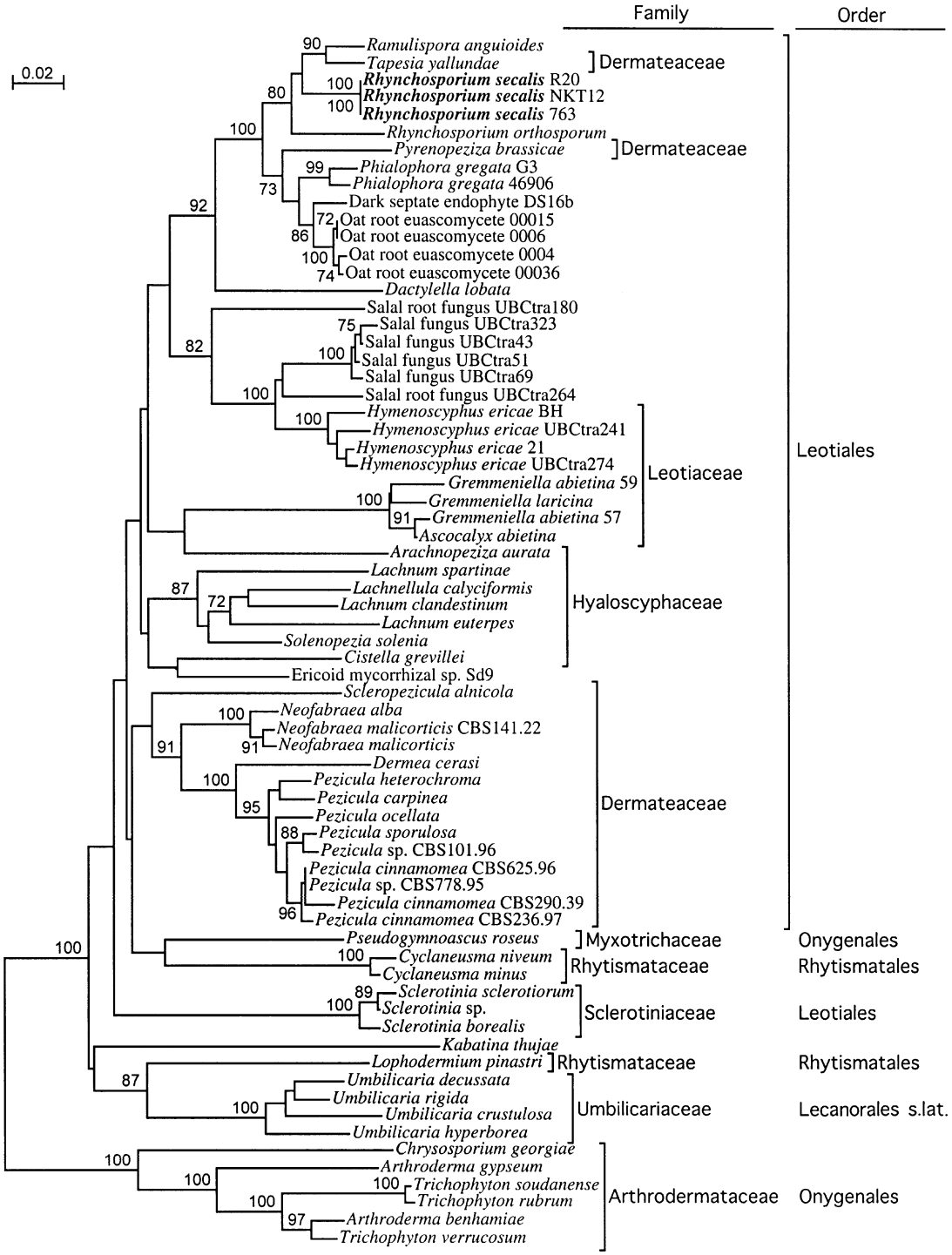

Fig. 3. Unrooted neighbour-joining tree of 76 sequences of the internal transcribed spacer region of the ribosomal DNA from *Rhynchosporium secalis* (indicated in bold) and other fungi. Sequences from several species in the *Arthrodermataceae* were included as an outgroup. All bootstrap values of 70 or greater (percent of 1000 replications) are indicated, rounded to the nearest integer. Families and orders of teleomorph genera are indicated by brackets to the right. Branch lengths are proportional to genetic distance, which is indicated by a bar at the upper left.

yallundae differed from that of *R. secalis* isolate 763 by 26 nucleotides. The next most closely related species with a known teleomorph was *Pyrenopeziza brassicae*. Both isolates of *P. brassicae* had an identical ITS sequence that differed from the *R. secalis* isolate 763 sequence by 57 nucleotides. The other ITS sequences that were closely related to those of *R. secalis* were from soybean and Adzuki-bean isolates of *Phialophora gregata*, several oat-root associated euascomycetes isolated by Carter *et al.* (1999), and a dark septate endophyte from *Ranunculus* (Fig. 2).

For the second analysis, duplicate sequences identified in the first analysis were removed. This eliminated four of the five identical *R. secalis* sequences, one of the

sequences of *Pyrenopeziza brassicae*, and the sequences of *Ramulispora aestiva* and *Tapesia acuformis* which were identical to that of *T. yallundae*. Sequences that differed by one or more nucleotides were retained. The complete alignment required 41 profile steps, 18 of which could be improved with minor manual editing.

The second analysis placed *R. secalis* in a much broader evolutionary context (Fig. 3). The *R. secalis* isolates clustered with the same taxa as in the first analysis with 100% bootstrap support. This group has teleomorphs, when known, in the *Dermateaceae*. The next most closely related cluster contained sequences of *Hymenoscyphus ericae*, a species in the *Leotiaceae*. Both the *Leotiaceae* and *Dermateaceae* are in the order *Leotiales*. ITS sequences of species from the *Hyaloscyphaceae* also were in the *Leotiales* cluster that included *R. secalis* (Fig. 3).

The *Leotiaceae* and *Rhytismataceae* in this analysis had members in very different clusters and clearly were polyphyletic. Similarly, the orders *Onygenales* and *Rhytismatales* were polyphyletic. All taxa in the *Leotiales* were included in a single large cluster (Fig. 3). However, this cluster had low bootstrap support and included members of the orders *Onygenales* and *Rhytismatales*.

DISCUSSION

The analysis of ITS sequences revealed clearly that the anamorphic ascomycete *Rhynchosporium secalis* is closely related to the teleomorph genera *Tapesia* and *Pyrenopeziza*. Both of these genera are discomycetes in the family *Dermateaceae* (Hawksworth *et al*. 1995) and clustered with *R. secalis* and several other anamorphic fungi with 100% bootstrap support. In addition to *R. secalis*, this monophyletic group included *R. orthosporum*, soybean and Adzuki-bean isolates of *Phialophora gregata*, a dark-septate endophyte, and several isolates of oat-root associated ascomycetes which were not identified to genus or species (Carter *et al*. 1999). On the basis of the ITS analysis it seems likely that the teleomorphs of all these species, if they exist, will be small, inoperculate discomycetes similar to *Tapesia* and *Pyrenopeziza*.

The close relationship of *R. secalis* with *Tapesia* was unexpected. The anamorph of the *Tapesia* species in these analyses is *Ramulispora* (Robbertse, Campbell & Crous 1995, Stewart *et al*. 1999). Species in this genus produce conidia that are five-to-seven celled (Wiese 1987), curved, and may be branched (Robbertse *et al*. 1995), which contrasts with the two-celled, unbranched conidia of *Rhynchosporium* (Caldwell 1937). Furthermore, conidia of *Ramulispora* species are produced from small sporodochia arising from substomatal stromata and have short conidiophores that may be simple or branched (Robbertse *et al*. 1995). None of these structures is produced by species of *Rhynchosporium*. Therefore, the close relationship between *Rhynchosporium* and *Ramulispora* could not have been deduced from analyses of morphological characters alone.

With hindsight, the symptoms caused by *Rhynchosporium* and *Ramulispora* are somewhat similar, and their host ranges overlap. The holomorphs *Tapesia yallundae* and *T. acuformis* cause eyespot disease of wheat, barley and other grasses (Robbertse *et al*. 1995, Wallwork & Spooner 1988). Eyespot symptoms are produced at the base of the wheat plants usually within 4 cm above or below the soil line (Wiese 1987). These lesions have a characteristic elliptical or 'eye' shape that is light in the centre with a dark margin (Wiese 1987), which is somewhat similar to the classic lens-shaped, dark-margined lesions caused by *Rhynchosporium secalis*. Eyespot lesions on barley are similar to those on wheat (Mathre 1982) and also are somewhat reminiscent of scald lesions. Species of *Ramulispora* formerly were classified in *Cercosporella* or *Pseudocercosporella* (Deighton 1973). However, on the basis of recent phylogenetic analyses all species of *Pseudocercosporella* infecting cereal crops have been transferred to *Ramulispora* (Robbertse *et al*. 1995, Stewart *et al*. 1999). These analyses of ITS sequences support the conclusion of Stewart *et al*. (1999) that species of *Ramulispora* are not related to *Cercospora* or other fungi with loculoascomycete teleomorphs.

On the basis of the ITS analyses, the teleomorph of *Rhynchosporium secalis*, if it exists, is predicted to be a species of *Tapesia*. The ITS sequences of *R. secalis* had fewer differences from those of the *Tapesia* species in the database than they did from that of the other species of *Rhynchosporium* tested, *R. orthosporum*. In fact, *R. secalis* clustered between *R. orthosporum* and *Tapesia* (Fig. 3), and all three formed a monophyletic group with high bootstrap support.

There are several potential explanations for why the teleomorph of *R. secalis* has not been found. One explanation could be that it simply does not exist, either due to the absence of suitable mating partners or because *R. secalis* has lost this ability during its evolution. However, based on analyses of associations among genetic markers in natural field populations, Salamati *et al*. (2000) proposed that *R. secalis* populations are recombining and that the teleomorph plays a significant role in the population biology of barley-infecting populations. Therefore, this explanation seems less likely. Another possible explanation is that the teleomorph may be formed on another, possibly unknown, host, but not on barley or other agronomic crops. A third potential explanation is that previous investigators simply did not know where or when to look, or what to search for. This last question now can be addressed. If *R. secalis* is closely related to species of *Tapesia* and *Pyrenopeziza*, its teleomorph should be a small apothecium produced directly on infected host tissue. Apothecia of *T. yallundae* are 0.4–1.5 mm diam (King 1991, Wallwork & Spooner 1988), sessile, and produced in groups from a hyphal mat (Wallwork & Spooner 1988). Those of *Pyrenopeziza brassicae* are

smaller (0.03–0.58 mm diam) and also produced directly on infected host tissue (Lacey, Rawlinson & McCartney 1987). Apothecia of *P. brassicae* can be found easily on dead tissue that remains damp following rain, but they shrink and become difficult to find when dry (Lacey *et al*. 1987). Apothecia of *T. yallundae* are found only on dead tissue several months after harvest (Dyer *et al*. 1994). Therefore, the teleomorph of *R. secalis* is most likely to be produced on dead leaf and stem tissue following rainy periods from 1–10 months after harvest of the crop. Knowing where, when, and what to look for may increase the chance of discovering a teleomorph for *R. secalis*.

Although these analyses identify species of *Tapesia* and *Pyrenopeziza* as close relatives of *R. secalis*, the taxonomy of this group remains uncertain. Hawksworth *et al*. (1995) consider *Tapesia* a synonym for *Mollisia*, and *T. yallundae* and *T. acuformis* currently are listed as species of *Mollisia* in the GenBank taxonomy database, even though neither species has been transferred formally into that genus. Aebi (1972) also concluded that *Tapesia* and *Mollisia* are synonymous, but considered *Tapesia* to be the valid name for this genus. For the current paper the most recent, validly published names for these species have been retained.

Higher-level classifications of these taxa are even more confusing. Both *Pyrenopeziza* and *Tapesia* are considered to be in the family *Dermateaceae* by Hawksworth *et al*. (1995). However, the ITS analysis revealed that they clustered separately from species of *Dermea*, *Neofabraea*, *Pezicula*, and *Scleropezicula* which are classified in the same family. Clearly, the *Dermateaceae* as defined by Hawksworth *et al*. (1995) is polyphyletic and probably should not contain *Pyrenopeziza* and *Tapesia*. The GenBank taxonomy database lists these species as belonging to the family *Mollisiaceae*, and this would make sense if species of *Mollisia* cluster with *Tapesia* and *Pyrenopeziza*. However, no sequences from other species of *Mollisia* are available from GenBank or other public sources so it is not possible to test this hypothesis currently. Obviously, much additional work is needed to clarify the taxonomic relationships of *Tapesia*, *Pyrenopeziza* and other species related to these taxa.

The inclusion of *Tapesia* and *Pyrenopeziza* species in the ITS database was serendipitous. None of these sequences was available in GenBank, so they were not identified in the BLAST searches. The *Tapesia* sequences were downloaded (as species of *Ramulispora*) from TreeBASE (http://herbaria.harvard.edu/treebase/) during an analysis for another study (Goodwin *et al*. 2001) and BLAST results on the *Ramulispora* sequences were recognised as being very similar to those for *Rhynchosporium secalis*. Existence of the unpublished *Pyrenopeziza* sequences was revealed during correspondence with Simon Foster on other matters, and they were requested because this genus is related to *Tapesia*. The sequence for *Rhynchosporium orthosporum* was found during a recent literature search. Reliance only on sequences deposited in GenBank would not have allowed resolution of *R. secalis* relationships down to probable teleomorph genus.

These analyses revealed that anamorph characters in this group do not reflect phylogenetic relationships as well as those of the teleomorphs. A similar conclusion was made recently for loculoascomycetes in the order *Dothideales* (Crous *et al*. 2000, Goodwin *et al*. 2001). This apparent rapid evolution of anamorph morphology may occur in many ascomycete groups and indicates that similarities or differences in anamorph characters do not always reflect past evolutionary history.

The phylogenetic analyses indicate that other higher-level classifications of the discomycetes also may need to be revised. In addition to the *Dermateaceae*, the *Leotiaceae* and *Hyaloscyphaceae* were polyphyletic, as was the *Rhytismataceae* in the *Rhytismatales*. *Pseudogymnoascus roseus* clustered with the *Leotiales* in both the 18S and ITS analyses, and may belong in that order rather than the *Onygenales*. The *Rhytismatales* also was polyphyletic; species of *Cyclaneusma* may belong in the *Leotiales* rather than the *Rhytismatales*. However, bootstrap support for many of the higher-level relationships often was low, probably due to the difficulty in aligning sequences at greater evolutionary distances. Correcting for multiple substitutions (Kimura 1980) and excluding positions with gaps made little or no difference in tree topology, particularly for clusters with high bootstrap support (data not shown). The 18S sequences were not as useful for making phylogenetic inferences, probably because fewer sequences were available and they contained a lower level of phylogenetically informative sites. Sequencing of additional genes will be required to test these hypotheses about relationships among families and orders within the discomycetes.

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